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Human tumors acquire a marked genomic instability as part of their progression from normal to malignant cells. This instability is likely due in part to the progressive shortening and transient loss of telomeres from chromosome ends. Loss of telomeres allows chromosomes to fuse end-to-end, triggering chromosome fusion-bridge-breakage cycles that lead to genome rearrangements, loss of heterozygosity, and gene amplification. The initial steps in chromosome fusion-bridge-breakage cycles are being studied by introducing site-specific double-strand breaks adjacent to interstitial telomere sequences in a marked region of a specially engineered test chromosome. A color-based detection system based on the green fluorescent protein is being developed to aid in detection of the early steps in fusion-bridge-breakage cycles. By varying the length of the telomere sequence, we will measure the length of telomere sequence that is required to protect chromosome ends and prevent genomic instability in breast cancer cells. A test chromosome is being constructed using the APRT locus in CHO cells, which will permit a measure of the effects of telomere sequence on the frequencies of chromatid fusion. Once the properties of the test chromosome are characterized in CHO cells we will transfer the engineered chromosome by microcell fusion into human breast cancer cells at different stages of tumor progression.

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INTRODUCTION

The progression of tumors from benign to malignant is often marked by genomic instability, resulting commonly in a highly rearranged genome. It has recently been shown that progressive shortening and transient loss of telomeres from the ends of chromosomes may contribute significantly to these genome rearrangements. Loss of telomeres allows chromosomes to fuse end-to-end, initiating cycles of chromosome fusion-bridge-breakage, which can result in genome rearrangements, gene amplification, and loss of heterozygosity. The purpose of the research supported by this grant is to define the lengths of telomere sufficient to prevent such rearrangements and to study the initial steps in chromosome fusion-bridge-breakage cycles. These processes will be investigated by introducing site-specific double-strand breaks adjacent to interstitial telomere sequences in a genetically marked region of a specially engineered test chromosome. Ultimately, the test chromosome, which will be developed in Chinese hamster ovary (CHO) cells, will be transferred into breast cancer cells at different stages of tumor progression to assess frequencies of fusion-bridge-breakage cycles in those cells.

In the first year of the grant, we demonstrated that double-strand breaks induced by expression of I-SceI could be healed by capture of a telomere-bearing plasmid. Through analysis of these events, we determined the orientation of the adenine phosphoribosyl transferase (APRT) gene on the chromosome, which was previously unknown. Next we performed experiments in which we used I-SceI to cleave adjacent to telomere sequence on the chromosome at the APRT locus. These experiments, which were performed in tetraploid cells, revealed that the rate of chromosome loss obscured any truncation events that might have occurred. This observation has necessitated a redesign of the test chromosome, which has been the major focus of our attention in the second year of the grant. In addition, we also began construction of a color-based system for detecting chromatid fusion.

In the current year of the grant, we have nearly completed construction of a redesigned targeting vector and have developed an appropriate cell line into which the new vector can be targeted. As described below, introduction of the new vector into these recipient cells will create a test chromosome that should be a very sensitive detector of chromosome truncation events. Specifically it has been designed so that neither chromosome loss nor homologous recombination will interfere with detection of the desired truncation events. In addition, our color-based detection system for chromatid fusion is ready to be inserted once the properties of the test chromosome have been verified.

BODY

Redesign of the test chromosome.

Our original test chromosome was derived from a design that was appropriate for detecting homologous and nonhomologous recombination events at the APRT locus (37,38), which was the initial focus of our work on telomere sequence. Work in the first year of this grant defined the orientation of the APRT locus on the chromosome, allowing us to explicitly represent the test chromosome as shown below.

Telomere---mutant 5'APRT---FRT---mutant 3'APRT---plasmid----TK----GPT---5'APRT---I-SceI/(CCCTAA)$_n$---FRT---3'APRT---Centromere

This test chromosome was generated by FLP/FRT (Flip Recombinase/ Flip Recombinase Target) site-specific recombination between the circular targeting vector and the recipient chromosome as shown below. This site-specific recombination event can be selected for because it generates an APRT+ cell.

TARGETING VECTOR   ---plasmid---TK---GPT---5'APRT---I-SceI/(CCCTAA)$_n$---FRT---mutant 3'APRT---

RECIPIENT CHROMOSOME   Telomere---mutant 5'APRT---FRT---3'APRT----Centromere

The resulting test chromosome carries a doubly mutant APRT gene nearer the telomere and a functional APRT gene nearer the centromere. Both these genes carry an FRT site in the middle of their second intron; the FRT site in this location does not interfere with gene expression. The functional, centromere-proximal APRT gene additionally carries the I-SceI recognition site adjacent to the seeding end of 800 base pairs of telomere sequence, also in the second intron; these sequences do not interfere with gene expression. Between the duplicated copies of the APRT gene are located two other selectable marker genes: the herpesvirus thymidine kinase (TK) gene and the bacterial guanine phosphoribosyl transferase (GPT) gene. These two genes have proven useful in various selections for recombinants. By selecting for APRT– cells, we can detect homologous recombination events between the duplicated APRT genes (which are the most common events), nonhomologous recombination events that disrupt the functional APRT gene, and mutation events that inactivate the functional APRT gene.

To extend the usefulness of this system for detecting chromosome truncation events, we prepared tetraploid cells to provide cover for any essential genes that might be located between the APRT locus and the telomere. This coverage is necessary because the APRT locus and surrounding chromosomal sequences are present in a single copy (haploid) in these CHO cells. Fusion was accomplished with a cell line that carried a defined deletion of the APRT locus. We anticipated that expression of I-SceI in these tetraploid lines would generate APRT– cells by chromosome truncation events, in addition to the other events listed above. We were particularly interested in the potential competition between homologous recombination events and chromosome truncation events. Would a double strand break adjacent to a telomere sequence preferentially generate homologous recombinants or chromosome truncation? This answer to this question was obscured by the unexpectedly high frequency of chromosome loss, an unanticipated outcome.

These results required a redesign of the test chromosome so that chromosome loss (a frequency of 10^{-3}) would not interfere with our ability to detect chromosome truncation events. In addition we wanted to eliminate repeated APRT sequences to prevent homologous recombination (a frequency of about 10^{-4}). Eliminating homologous recombination and chromosome loss would lower the background events (derived from mutation and nonhomologous recombination) to less than 10^{-5}. The design of the test chromosome that meets these requirements is shown below.

TEST CHROMOSOME   Telomere---FRT---plasmid---TK---I-SceI/(CCCTAA)$_n$---5'APRT---GPT---FRT---3'APRT---Centromere

This test chromosome has the desired features. It carries no duplicated APRT sequences so that the potential for homologous recombination is eliminated. A chromosome truncation event can be selected for as a TK–GPT+ cell. Chromosome loss will not be detected because it would generate TK–GPT– cells. In addition, the test chromosome could be used without further modification for transfer into breast cancer cells that have been rendered mutant for hypoxanthine-guanine phosphoribosyl transferase (HPRT), which is a straightforward procedure. Construction of this test chromosome requires the targeting vector and recipient chromosome shown below.

TARGETING VECTOR   ---plasmid---TK---I-SceI/(CCCTAA)n---5'APRT---GPT---FRT---
                                                    X
RECIPIENT CHROMOSOME   Telomere---FRT---3'APRT---Centromere

In order to create the desired test chromosome, we needed to construct an appropriate, new targeting vector and recipient chromosome.

Construction of the recipient chromosome.

The recipient chromosome was constructed in several steps. First, a targeting vector that contained a precise deletion of the 5′APRT sequences (but retained sequences upstream of the APRT locus) was generated using appropriate flanking restriction sites. Next, it was introduced into a modified, functional APRT locus by site-specific recombination to generate an APRT– modified chromosome, as shown below.

TARGETING VECTOR ---plasmid---TK---upstream APRT---FRT---mutant 3′APRT—

                                                   X

CHROMOSOME Telomere---upstream APRT---5′APRT---FRT---3′APRT---Centromere

MODIFIED CHROMOSOME
Telomere---upstream APRT---5′APRT---FRT---mutant 3′APRT---plasmid---TK---upstream APRT---FRT---3′APRT---Centromere

Finally, the desired recipient chromosome was identified among TK– cells, some of which had undergone homologous recombination between the duplicated upstream APRT sequences to give the appropriate chromosome structure.

RECIPIENT CHROMOSOME Telomere---upstream APRT---FRT---3′APRT---Centromere

(Note that this recipient chromosome is identical to the one shown in the previous section. In this diagram, the upstream APRT sequences are explicitly shown because they are relevant to how the chromosome was generated. In the diagram in the previous section, the upstream APRT sequences are present, but not shown for simplicity because they are irrelevant to the proposed experiments.)

Construction of the targeting vector.

Repositioning the selectable markers and eliminating sequences from the APRT locus other than the 5′ half of the APRT gene required that we start over from scratch; we could not simply modify any existing targeting vector. In our design for the test chromosome, we wanted to move the selectable GPT gene as far away from the site of the truncation to insulate it from potential silencing effects caused by proximity to the new telomere created by truncation. The most distant location compatible with delivery by the targeting vector is adjacent to the FRT in the second intron of the APRT gene, as shown above. This design, however, requires that both the APRT gene and the GPT gene can function when they are arranged as overlapping transcription units. Since we had never tested another complete transcription unit inside the APRT gene, we did not know whether this arrangement was viable. To test it we cloned the GPT gene, in both orientations, adjacent to the FRT site in an otherwise wild-type APRT gene. These constructs were transfected into APRT– cells and parallel cultures were selected for either APRT+ colonies or GPT+ colonies. If there was any problem with overlapping transcription units, then APRT+ colonies, or GPT+ colonies, or both should be low. The results of these transfections revealed that both genes are functional regardless of the orientation of the GPT gene: APRT+ colonies and GPT+ colonies were obtained in equal numbers at high levels.

Having verified this element of the proposed targeting vector, we finalized a strategy for its construction. We designed a polylinker into which the various elements of the targeting vector could be cloned in a particular order so that there was minimum interference of one cloning step with the next. The resulting 70-nucleotide polylinker was constructed from overlapping shorter segments, inserted into a convenient plasmid backbone, and sequenced to verify its structure. The TK gene, the I-SceI/(CCCTAA)$_n$ fragment, and the 5′APRT—GPT—FRT fragment were isolated from other vectors and inserted one-at-a-time into sites in the polylinker. The structure of the final targeting vector is now being

verified. Once verified, the targeting vector will be site-specifically recombined into the recipient chromosome to generate the test chromosome.

Construction of the color-based detector of chromatid fusion events.

By the end of the first year of the grant, we had inserted an artificial intron into the middle of the gene for the green fluorescent protein (GFP). Tests of that construct for ability to express GFP, which would require correct splicing of the added intron, were negative: only very low-level expression of GFP was obtained. In parallel studies for research supported by other grants, we had a corresponding difficulty generating artificial, functional exons. It seems that consensus-splicing signals are not all that is required for accurate splicing. In those studies we ultimately solved our problems and developed functional artificial exons (but it was a very time-consuming process). For the purposes of this grant, however, we realized that an alternative approach should work equally as well as the one originally proposed; namely, to fuse GFP to another gene that already had introns. Such a fusion gene could be used in the way originally proposed. By inverting the initial exons we would eliminate GFP expression, and a chromatid fusion event adjacent to such a gene would relink the initial exons from one chromatid with the distal exons from the other chromatid, thereby turning on expression of the green fusion protein. This alternative strategy occurred to us because we had just created (for other purposes) a C-terminal fusion of the human genomic rhodopsin gene with GFP. The fused gene is under control of the CMV promoter and upon transfection into cells (or from a chromosomal site of integration) it expresses brightly green. We intend to use this available, well-characterized rhodopsin-GFP fusion to serve as our color-based detector of chromatid fusion. (Note that the polylinker in the redesigned targeting vector has appropriate sites where the rhodopsin-GFP fusion can be inserted in order to generate an appropriate test chromosome to follow chromatid fusion.) When the properties of the redesigned test chromosome have been verified, we will insert the rhodopsin-GFP fusion gene in the form designed to detect chromatid fusion.

CONCLUSIONS

In this grant period we have focused on construction of the redesigned test chromosome. We have in hand an appropriately modified recipient chromosome and have nearly completed construction and verification of the targeting vector. The necessity for a complete redesign of the test chromosome has significantly delayed our completion of the original objectives of this grant. Nevertheless, the development of a well-characterized test chromosome that can be used to detect chromosome truncations is well worth the considerable effort involved in its construction. Once its usefulness is verified, it can be applied to questions posed in the original grant.

Our objective for the third year of this grant are listed below:

Objective 1: Verify structure of targeting vector. (*month 1*)

Objective 2: Create and verify structure of the test chromosome. (*months 2-3*)

Objective 3: Transfer the test chromosome into APRT– CHO cells by microcell fusion and verify the presence of the test chromosome. (*months 4-6*)

Objective 4: Express I-SceI in hybrid cells and select for TK–GPT+ colonies. (*month 7*)

Objective 5: Analyze the structure of the test chromosome in TK–GPT+ colonies. (*months 8-9*)

If, as we anticipate, the test chromosome is truncated and a new telomere is seeded, we will transfer the test chromosome into HPRT– breast cancer cells and verify that it can be truncated in such cells. That will lay the foundation for the set of experiments we posed in the original grant. In addition, we will begin the process of inserting the color-based detector of chromatid fusion into the targeting vector for generation of an appropriate test chromosome. These studies will extend beyond the lifetime of this grant, but they are important and novel enough that we anticipate that we will be able to obtain support for their continuation.

KEY RESEARCH ACCOMPLISHMENTS

- Redesign of test chromosome

- Near-final construction of targeting vector for generating test chromosome

- Construction of recipient chromosome for integration of targeting vector to create test chromosome

- Construction of a GFP fusion to serve as color marker for detecting chromatid fusion

REPORTABLE OUTCOMES

- None as yet